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Name of Subcontractor: Lynne P. Rutzky, Ph.D.

Title: Associate Professor of Surgery

Institution: University of Texas Health Science Center at Houston

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Title: Director of Sponsored Projects

Phone: 713/500-5847

Fax: 713/500-5848

Type I insulin-dependent diabetes mellitus (IDDM) remains a major cause of morbidity and mortality in both pediatric and adult populations, despite significant advances in medical management. While insulin therapy treats symptoms of acute diabetes, it fails to prevent chronic complications such as microvascular disease, blindness, neuropathy, and chronic renal failure. Strict control of blood glucose concentrations delays but does not prevent the onset and progression of secondary complications (1,2). Although, whole pancreas transplantation restores physiological blood glucose levels, a continuous process of allograft rejection causes vascular and exocrine-related complications (4). Recent advances in methods for isolation and purification of pancreatic islets make transplantation of islet allografts an attractive alternative to whole pancreas transplantation (3,6). However, immunosuppressive drugs are necessary to prevent rejection of islet allografts and many of these drugs are known to be toxic to the islets. Since auto-transplants of isolated islets following total pancreatectomy survive and function *in vivo*, it is apparent that a major obstacle to successful clinical islet transplantation is the immunogenicity of the islet allografts (5).

The Rotating Wall Vessel (RWV) bioreactor technology developed by NASA offers a unique opportunity to culture cells in simulated microgravity conditions. Solid body rotation simulates microgravity conditions by randomizing the gravitational vector in a configuration in which zero headspace, horizontal rotation, and membrane oxygenation simulate the buoyancy effects of microgravity (7). Cells are cultured in continuous free fall through the medium and are exposed to an environment characterized by low shear stress, low turbulence, and high mass transfer. Many cell types cultured in RWV bioreactors grow as three dimensional organoid structures with tissue-like organization and function (8-12).

The present studies evaluated the *in vivo* survival of mouse or rat pancreatic islet allografts cultured in bioreactors, or in static Petri dish cultures, in comparison with freshly-isolated islets. There was no standard immunosuppression used in these studies. In the rat model, syngeneic Lewis (LEW; RT1^l), or allogeneic Buffalo (BUF; RT1^b) or Brown Norway (BN; RT1ⁿ) islets were transplanted to LEW recipients either under the kidney capsule or embolized in the liver via the portal vein. Following enzymatic digestion of the pancreas with collagenase, islets were isolated by discontinuous dextran gradient centrifugation (13). Islets separated manually from acinar tissue fragments were enumerated under a dissecting microscope. Purified donor islets (LEW, BUF, or BN) were used immediately for transplantation (freshly-isolated) or were cultured either in stationary Petri dishes (37 C; 5% CO₂) or in reusable HARV or STLV RWV (50 mL) bioreactors (37 C; 5% CO₂) with a continuous rotation at 12-14 revolutions/minute (RPM). Rat islets were cultured in CMRL1066 Medium (GIBCO), supplemented with 1% L-glutamine, 1% MEM vitamins, 1% HEPES buffer, 10% fetal bovine serum (Hyclone), and antibiotics. CMLR 1066 medium containing 10% fetal bovine serum (FBS) is a better culture medium than RPMI 1640 with 10% FBS for culturing rodent islets. Direct microscopic examination showed that islets cultured in CMRL 1066 medium had fewer islets adhering to the Petri dish surface. In addition, islets appeared brighter with less darkening—suggesting reduced central necrosis. Aliquots of fresh and cultured islets were routinely stained with dithizone (14;15) to visualize insulin content. Samples were prepared routinely for hematoxylin and eosin staining and light microscopic examination of morphology. Following transplant, blood glucose levels were monitored at intervals to evaluate the efficacy of the transplant to cure diabetes. Two consecutive blood sugar levels greater than 300 mg/dL glucose were considered a rejection endpoint.

To increase total number of rat islets, bioreactors were inoculated twice (at days 1 and 3) with islets cultured previously in Petri dishes. Islets were cultured in bioreactors an average of 11 ± 2 days. In the syngeneic rat transplant model, both Petri dish stationary cultured and bioreactor cultured LEW islets were capable of curing diabetes in streptozotocin-treated diabetic LEW

recipients, as documented by a graft mean survival time (MST) of 95 ± 10 days ($n=2$) and 100 ± 0 days ($n=2$), respectively. In the allogeneic rat transplant model, euglycemia (<300 mg glucose/dL) was maintained by Petri dish-cultured BUF or BN islets in LEW recipients at 17.5 ± 3.5 days ($n=2$) and 30 days ($n=1$), respectively. Similarly, the bioreactor-cultured BUF or BN islets maintained euglycemia in LEW recipients for 15 days ($n=1$) or for 20 days ($n=1$), respectively. Although the rat islet model was successful, we performed the majority of experiments in mice to increase the yield of islets.

In the allogeneic model, C57BL/10 (H-2^b) pancreatic islets were transplanted under the kidney capsule into streptozotocin-treated diabetic C3H (H-2^k) recipients. Streptozotocin-treated mice with glucose level of 350 mg/dL in two consecutive measurements were considered diabetic. Mouse islets were isolated using a slight modification of the rat method. Purified C57BL/10 islets were immediately transplanted (freshly-isolated) or they were cultured in either stationary Petri dishes (37°C, 5% CO₂), or in RWV bioreactors (37°C, 5% CO₂) with a continuous rotation at 12-14 RPM. Islets were cultured in CMRL 1066 medium as described above. Some euglycemic animals with long term islet transplants were nephrectomized and blood sugar levels were followed at intervals for 1-2 weeks to determine whether the transplanted islets were required to maintain normal blood sugar levels.

Culture experiments showed that fibroblastic and other adherent cells present in fresh preparations were removed by transfer to new Petri dishes during the first week of culture. Such procedures should eliminate the antigen presenting cells (APC) and lymphocytes (passenger cells) that express class II major histocompatibility complex (MHC) antigen. Although class I MHC antigens are expressed on islets and passenger cells, class II MHC antigens are expressed only on passenger cells. The glucose concentration (Glucose 2 Analyzer, Beckman Instruments, Fullerton, CA) has been monitored in culture medium of islets cultured in Petri dishes and in various conditions in bioreactors. Cultured islets were metabolically quiescent, as shown by intact glucose and stable pH levels during one week of culture. Interestingly, Petri dish-cultured islets containing fibroblastic cells consumed glucose and displayed lower pH levels. A portable clinical analyzer (Princeton, NJ) confirmed these observations. Insulin, as measured by radioimmunoassay, was not detectable in medium of islets cultured with 100 mg glucose/ml concentration.

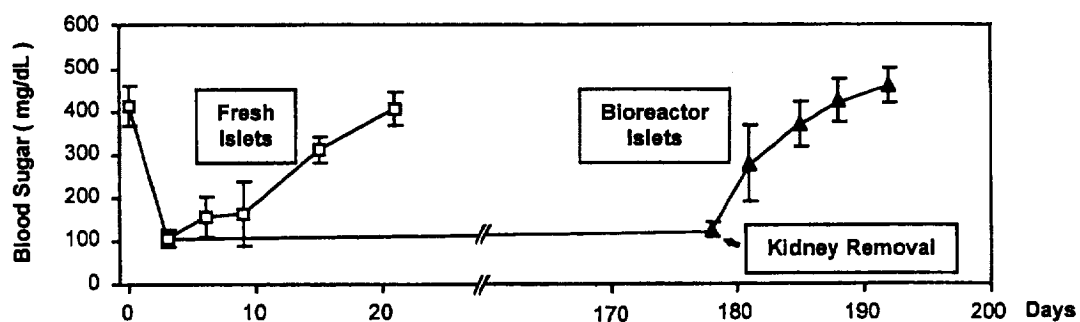


Figure 1. Glucose levels in recipients transplanted with bioreactor-cultured allogeneic islets.

The *in vivo* results showed that allogeneic mouse pancreatic islets cultured in bioreactors for seven days prior to transplantation under the kidney capsule cured diabetes for more than 200 days ($N=5$) in comparison to only 11.9 ± 0.9 days ($N=5$) in recipients transplanted with freshly isolated islets (Fig. 1). Removal of kidneys with transplanted islets following 200 days of transplantation caused recurrence of diabetes. Following transplantation of various islets preparations, blood glucose levels were measured twice a week and graft failure was assumed after two consecutive blood glucose readings greater than 300 mg/dL. Freshly-isolated C57BL/10

allogeneic islets were acutely rejected as confirmed by the glucose level of 453 ± 66.6 mg/dl on day 11 postgrafting (n=8). In contrast, islets cultured in a bioreactor survived for more than a MST of 170 days (range 150-220 days; n=6) without any signs of allograft rejection. Only after the kidneys with islet allografts were removed, did the recipients develop diabetes with a glucose levels of 371 ± 50 mg/dl on day 177 and of 426 ± 50 mg/dl on day 180 postgrafting (n=5). These results document that islets cultured in microgravity environment lost their immunogenicity or/and become more resistant to the non-specific and alloantigen-specific destruction. In addition, these results strongly suggest that islets cultured in bioreactors are significantly less immunogenic in comparison to freshly isolated islets. Therefore, bioreactor-cultured islet allografts may be transplanted without any immunosuppression to potentially induce a state of transplantation tolerance. Furthermore, morphological analysis documented that islets cultured in bioreactors are superior without central necrosis often observed in islets cultured in stationary Petri dishes. In addition, our preliminary results suggest that fewer bioreactor-cultured islets than stationary Petri dish-cultured islets were necessary to produce euglycemia (<300 mg glucose/dL).

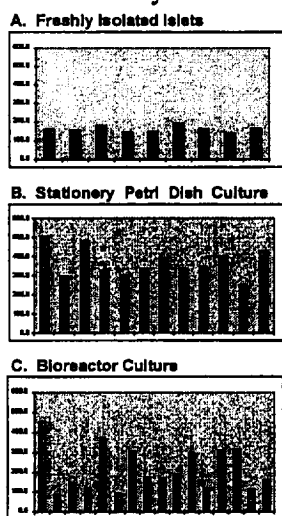


Figure 2. Size of islets cultured in bioreactor

In our studies, stationary Petri-dish (60 x 15 mm) mouse islet cultures were initiated with ≈ 300 -700 freshly-isolated islets in 4 ml CMLR 1066 medium, there were ≈ 200 islets left after 7-day culture. Bioreactor culture of islets appears to enhance islet aggregation. However, even with variable aggregation, we found that as few as 20 islets cultured in bioreactor were sufficient to cure diabetes for over 100 days in allogeneic transplants under the kidney capsule, whereas 500 fresh allogeneic islets were necessary to prevent diabetes for a short time (11.9 ± 0.9 days (N=5)). The bioreactor culture usually started with ≈ 1000 mouse islets in 50 ml total volume, and ended with ≈ 300 mixed individual and clustered islets. The EIN conversion (13) was used to obtain a uniform equivalent number of freshly-isolated C57BL/10 islets and islets cultured in bioreactors for transplantation under the kidney capsule into diabetic syngeneic C57BL/10 recipients. The diameters of islets were measured in each group (freshly-isolated, stationary-cultured or bioreactor-cultured) using an ocular micrometer and a phase contrast microscope. The islets were evaluated accordingly with their sizes, namely they were subdivided into diameter classes every 50 μ m between 50-350 μ m with one class for islets larger than 350 μ m in diameter.

Furthermore, the equivalent islet number (EIN) was calculated for each group using a formula based upon cell volume to convert the different size islets into an equivalent with constant 150 μ m islet diameter (13). In particular, the aliquot for each preparation requires 250 equivalent islets of 150 μ m diameter islets (Fig. 2). Freshly-isolated islets displayed very consistent size of approximately 150 μ m in all experiments (Fig. 2A). In contrast, Petri-dish-cultured islets became smaller in size after 7-days, as documented by the EIN values (Fig. 2B). However, islets cultured in bioreactor had a variety of sizes, probably because of aggregation of islets in some experiments (Fig 2C). Since, the average size of a fresh islet is 150 μ m, all those above 350 μ m in culture include the aggregates of two or more islets.

In conclusion, our present experiments revealed that bioreactor may be used to culture pancreatic islets for transplantation. Furthermore, short 7-day culture of islets in bioreactor reduces significantly their immunogenicity as documented by long-term allograft survival. Finally, culture in bioreactor selects strongest islets as documented by limited number of islets necessary to cure diabetes.

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